

Characterization of the Lymphocyte Activation Gene 3-Encoded Protein. A New Ligand for Human Leukocyte Antigen Class II Antigens

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Summary

The lymphocyte activation gene 3 (LAG-3), expressed in human activated T and natural killer (NK) cells, is closely related to CD4 at the gene and protein levels. We report here the initial characterization of the LAG-3-encoded protein. We have generated two monoclonal antibodies after immunization of mice with a 30-amino acid peptide that corresponds to an exposed extra loop region present in the LAG-3 immunoglobulin-like first domain. The reactivity of these reagents is directed against LAG-3 since they recognize both membrane-expressed and soluble recombinant LAG-3 molecules produced in a baculovirus expression system. The two antibodies are likely to react with the same or closely related epitope (termed LAG-3.1) exposed on the LAG-3 first domain extra loop, as assessed in competition experiments on LAG-3-expressing activated lymphocytes. Cellular distribution analysis indicated that the LAG-3.1 epitope is expressed on activated T (both CD4⁺ and CD8⁺ subsets) and NK cells, and not on activated B cells or monocytes. In immunoprecipitation experiments performed on activated T and NK cell lysates, a 70-kD protein was detected after SDS-PAGE analysis. 45-kD protein species were also immunoprecipitated. Both the 70- and 45-kD proteins were shown to be N-glycosylated. In Western blot analysis, only the former molecule was recognized by the anti-LAG-3 antibodies, demonstrating that it is LAG-3 encoded. These anti-LAG-3 antibodies were used to investigate whether the LAG-3 protein interacts with the CD4 ligands. By using a high-level expression cellular system based on COS-7 cell transfection with recombinant CDM8 vectors and a quantitative cellular adhesion assay, we demonstrate that rosette formation between LAG-3-transfected COS-7 cells and human leukocyte antigen (HLA) class II-bearing B lymphocytes is specifically dependent on LAG-3/HLA class II interaction. In contrast to CD4, LAG-3 does not bind the human immunodeficiency virus gp120. This initial characterization will guide further studies on the functions of this molecule, which may play an important role in immune responses mediated by T and NK lymphocytes.

We have recently found (1) a novel member of the immunoglobulin superfamily (IgSF)² (2), termed lymphocyte activation gene 3 (LAG-3), that is selectively transcribed in human activated T and NK cells. The LAG-3 cDNA

encodes a 470-amino acid (aa) membrane protein with four extracellular IgSF domains. Analysis of the LAG-3-encoded aa sequence revealed notable patches of identity with stretches of sequences found at the corresponding positions in CD4. For example, the very unusual W × C sequence is found in domains 2 and 4 of both LAG-3 and CD4, while it is not present in an equivalent position in any other IgSF domains. In addition, as in the CD4 structure (3), there are some internal sequence homologies in the LAG-3 molecule between domains 1 and 3, as well as between domains 2 and 4, suggesting that LAG-3 has evolved like CD4 by gene duplication from a preexisting two-IgSF structure. Furthermore,

¹ Contributions of these authors were equivalent, and their order should be considered arbitrary.

² Abbreviations used in this paper: aa, amino acid; IgSF, immunoglobulin superfamily; LAG-3, lymphocyte activation gene 3; NS, nonspecific.

the characterization of genomic clones encompassing the LAG-3 locus (eight exons that span 6 kb) revealed that the position of the introns in the LAG-3 structure is very close to that of CD4, and that both genes include an intron within the first IgSF domain (1). Finally, both genes are located on the distal part of the short arm of chromosome 12 (band p13.3 for LAG-3; reference 1). Together, these structural considerations at the gene and protein levels supported the view that LAG-3 and CD4 are closely related and derive from a common four-domain evolutionary ancestor (1). LAG-3 and CD4 can therefore be regarded as evolutionary "first cousins" within the IgSF.

A unique feature of the LAG-3 molecule is the presence of a 30-aa extra loop sequence in the first NH₂ terminus Ig-like domain between β strands C and C'. Such a long extra loop structure has not been described in IgSF molecules. We have postulated that this extra loop does not disrupt the core of the IgSF fold that consists of β strands A, B, E and G, F, C, and therefore may be exposed to the solvent outside the hydrophobic Ig fold (1).

Based on this hypothesis, we have generated antibodies by immunizing animals with a 30-aa peptide corresponding to the extra loop aa sequence. These reagents recognize specifically both natural and recombinant LAG-3-encoded proteins and have been used to study the cellular distribution, the molecular structure, and the biological function(s) of the LAG-3 protein. In particular, since CD4 has been previously shown to interact with HLA class II molecules (4–8) and to be a receptor for HIV retroviruses (9, 10), we have examined the ability of the LAG-3 protein, transiently expressed in COS-7 cells, to bind these ligands.

Materials and Methods

Cell Cultures. PBMC were obtained from healthy donors by Ficoll-Hypaque density gradient centrifugation. Activated T cells were generated by stimulating PBMC at a cellular concentration of 2×10^6 /ml with 2 μ g/ml PHA-P in the presence or the absence of rIL-2 (250 U/ml). Activated B cells were generated by stimulation of 2×10^6 PBMC/ml with 0.3% (wt/vol) PWM. For monocyte activation, PBMC were first submitted to adherence in DMEM, 10% FCS on plastic flasks at 37°C. Adherent cells were recovered in RPMI supplemented with 1% FCS and incubated overnight with 5 μ g/ml LPS at 37°C.

Long-term cultured IL-2-dependent clonal or polyclonal T cells, including MBA8, T2, R2, PH28, 1C1 (CD3⁺ TCR- α/β ⁺), TH6-4, BK (CD3⁺ TCR- γ/δ ⁺), as well as the NK cell lines F55IIIIE5 and SIB5 (CD3⁻CD56⁺), were obtained from healthy donors and cultivated in the presence of rIL-2 on a feeder layer of irradiated EBV-transformed B cells (LAZ 388) as previously described (11, 12). *Spodoptera frugiperda* insect cells (Sf9 cells) and COS-7 cells were cultivated following standard protocols.

mAbs and Recombinant gp120 Protein. The reactivity of the mAbs was studied by indirect immunofluorescence, and samples were analyzed on an Elite Epics cytometer (Coulter Electronics, Inc., Hialeah, FL). 5,000 cells were analyzed in each sample. The mAbs used in these studies were anti-NKTa, which recognizes a TCR- α/β clonotypic determinant (13), anti-CD3 (OKT3), anti-CD4 (OKT4), anti-CD8 (OKT8), anti-CD71 (OKT9) (Ortho Diagnostics, Raritan, NJ), anti-CD56 (NKH1), anti-CD25 (IL-2R1)

(Coulter Immunology), and an anti-CD2 (XC3) produced in our laboratory (unpublished data). The binding of these antibodies were detected with either fluorescein- or PE-conjugated goat anti-mouse antibodies. The CD4-specific mAb OKT4E was provided by P. Rao (Johnson Pharmaceutical Research Institute, Raritan, NJ). The HLA-DR-specific D1.12 mAb (14) was a gift from R. Accolla (Ludwig Institute for Cancer Research, Lausanne, Switzerland). The 9.49 mAb (Coulter Immunology) is specific for the three HLA class II isotypes (15), 25.3.1 (Immunotech, Marseille, France) for LFA-1 and CD11a, and 8F5 for ICAM-1 (16). Purified soluble recombinant gp120 was kindly provided by M. P. Kiény and J. P. Lecocq (Société Transgène, Strasbourg, France).

Expression of LAG-3 Recombinant Proteins. A variety of eukaryotic genes, including CD4 (17), have been expressed in *Spodoptera frugiperda* insect cells infected with a recombinant Autographa californica nuclear polyhedrosis virus (AcMNPv), and the corresponding recombinant proteins have been shown to be functionally, antigenically, and biochemically similar to their natural counterparts (reviewed in reference 18). In the present experiments, a membrane as well as a soluble LAG-3 recombinant protein were produced using a baculovirus expression system, as described previously for the LAG-1 protein (19). Briefly, the first construction, designated LAG-3C (the complete coding region of LAG-3), included a 1,620-bp Eco47III-BglII fragment derived from the LAG-3 cDNA sequence, termed FDC (1), which was inserted into the BamHI site of the pVL941 vector. The second construction, designated LAG-3S, included an 1,106-bp Eco47III-BamHI fragment of sequence FDC and corresponds therefore to the first three Ig-like domains of LAG-3 (aa 1–363). Insect Sf9 cells were cotransfected with both the corresponding recombinant vector (LAG-3C/pVL941 or LAG-3S/pVL941) and the *Autographa californica* wild-type baculovirus genome (AcMNPv). After in vivo recombination, virus was recovered from the Sf9 supernatant and purified by limiting dilution using the FDC sequence probe.

The 1,620-bp Eco47III-BglII FDC fragment was also cloned into the CDM8 eukaryotic expression vector (gift from B. Seed, Massachusetts General Hospital, Boston, MA) at the BstXI sites after addition of BstXI linkers (Invitrogen, San Diego, CA). Small-scale DNA preparations of transformants in *Escherichia coli* MC1061/p3 were digested by SacI to determine the correct orientation of the cloned fragment. One clone in the reverse orientation, termed GAL, was used as a negative control. Transfection of COS-7 cells were performed by the DEAE-dextran method as previously described (8). For flow cytometry analysis on an Elite Epics, transfected cells were detached from plates with 1 mM EDTA in PBS and stained with the purified 17B4 mAb at 2 μ g/ml, and with a 1:50 dilution of FITC-conjugated goat antibody to mouse Igs (Nordic, Tilburg, The Netherlands).

For the immunoprecipitation analysis, COS-7 cells were transfected with the CDM8-LAG-3 plasmid by electroporation using a Cellject apparatus (Eurogentech, Liege, BE). COS-7 cells were electroporated (200 V, 1,500 μ F, and infinite shunt resistor) with 30 μ g/ml of plasmid in PBS at 10^7 /ml.

Production of Anti-LAG-3 Antibodies. A 30-aa peptide (GPPAAA-PGHPLAPGPHPAAPSSWGPRPRRY) designated 208B, which includes the extra loop protein sequence (1), was synthesized and coupled at its NH₂ terminus to the tetanus anatoxin carrier protein. Rabbit immunizations were performed by injecting subcutaneously 200 μ g of 208B-coupled peptide resuspended in CFA. Boost injections in IFA were administered at 4-wk intervals. Biozzi mice were immunized by an initial intraperitoneal injection of 50 μ g of 208B peptide in CFA, followed by injections on days 15, 30, and 50 in IFA. On day 70, mice were boosted via intravenous in-

jection of 20 µg of peptide in NaCl solution (0.9%), and 3 d later immunized splenocytes were fused with NS1 HAT-sensitive myeloma cells.

Hybridoma supernatants reactive on both LAG-3 surface-expressing Sf9 cells and PHA-activated T cells (PHA blasts) were selected by indirect immunofluorescence and subsequently subcloned in HAT medium supplemented with 10% FCS. The 17B4 mAb (IgG1) was purified from the hybridoma supernatant on protein A-Sepharose CL-4B Fast Flow column (Pharmacia LKB, Uppsala, Sweden).

The fine specificity of the anti-LAG-3 mAbs was determined by incubating at 4°C a nonsaturating dilution of the mAb with different concentrations of either the 30-aa 208B peptide or a 28-aa LAG-3-unrelated peptide (termed nonspecific [NS]), followed by indirect immunofluorescence on PHA blasts.

Western Blot Analysis. Cell lysates or supernatants were analyzed on a 10% polyacrylamide gel and proteins were transferred to a 0.1-µm nitrocellulose membrane using a MilliBlot-SDE electroblotting apparatus (Millipore, Bedford, MA). Antibody-reactive proteins were revealed according to the enhanced chemiluminescence kit (Amersham International, Amersham, UK). Briefly, the membrane was incubated with the specific antibody for 2 h at room temperature. The membrane was subsequently washed and then incubated for 1 h with a second antibody, either a peroxidase goat anti-mouse IgG (H+L) (Nordic) or peroxidase-conjugated swine anti-rabbit Igs (Dako, Glostrup, Denmark). The membrane was then washed and incubated for 1 min in a solution containing the detection reagent and autoradiographed.

Immunoprecipitation of the Antigens. Cell surface labeling using ¹²⁵I was performed by a standard lactoperoxidase method (20), and immunoprecipitations were carried out as previously described (21). Briefly, cell pellets were resuspended in 1 ml of lysis buffer at pH 7.2 containing 10 mM sodium phosphate, 1% triton X-100, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM PMSF, 1 mg trypsin inhibitor, 1 mM iodoacetamide, and 0.1% sodium deoxycholate. Extensive preclearings were carried out and specific immunoprecipitations were performed sequentially with antiactin (an IgG1 kindly given by Dr. Haynes, Duke University, Medical Center, Durham, NC), anti-NKTα (an IgG1 used as negative control), 17B4 (IgG1), and finally the XC3 anti-CD2 mAb. All the mAbs were coated to protein G-Sepharose beads and incubations with cell lysates were performed overnight at 4°C. After washing the beads in lysis buffer, samples were eluted by boiling in SDS sample buffer in the absence or in the presence of 5% 2-ME, and analyzed in 10% SDS-PAGE under nonreducing or reducing conditions. Gels were stained with coomassie blue, dried, and autoradiographed at -80°C.

N-Deglycosylation Treatment of Glycoproteins. After autoradiographic detection, the immunoprecipitated labeled proteins were excised, eluted, and lyophilized. The recovered material was rehydrated in 10 µl of 0.5% SDS buffer and denatured by boiling for 4 min. Digestion with 0.2 U of N-glycosidase F (PNGase-F; Boehringer, Mannheim, Germany) was carried out overnight at 37°C in a 40 µl buffer (pH 7.5) containing 250 mM sodium phosphate, 10 mM EDTA, 10 mM 2-ME, and 1% NOG (*n*-Octyl β-D-glucopyranoside). Cleavage products were resuspended in an equal volume of SDS sample buffer containing 5% 2-ME and analyzed by SDS-PAGE.

B Lymphocyte Adhesion to Transfected COS Cells. Rosette formation between transfected COS cells and B lymphocytes has been described in detail (8). Briefly, 5 × 10⁶ B lymphocytes were incubated for 1 h at 37°C with a COS cell monolayer in 12-well plates, 48 or 72 h after transfection with the recombinant LAG-3-

and GAL-CDM8 vectors, and then washed five times. After rosette formation, COS cells were stained with an indirect immunoperoxidase method, as described (8), using the LAG-3-specific 17B4 antibody. Observation was done under a light microscope. Rosette quantification was expressed as the percentage of LAG-3-positive cells that have retained five B lymphocytes or more. Inhibition of rosettes was performed by preincubating cells for 30 min with different concentration of mAbs. Incubation of Raji cells with anti-HLA-class II antibodies was done on cells fixed by incubation with 0.5% paraformaldehyde for 10 min at room temperature, followed by a two-time extensive washing in PBS and a 30-min incubation in DMEM 10% FCS. Rosettes were allowed to form in the presence of the inhibitors. Results are expressed as the rosette inhibition percentage of two or more experiments ± SD, calculated as follows: 100 × [1 - (% rosette with inhibitor / % rosette without inhibitor)].

Results

Generation of Anti-LAG-3 Antibodies. Rabbits and mice were immunized with the tetanus toxoid-coupled 208B peptide (see Materials and Methods), whose sequence is derived from the extra loop of the LAG-3 Ig-like first domain (1). Two murine hybridoma supernatants, designated 17B4 (IgG1) and 4F4 (IgM), were selected upon their reactivity in immunofluorescence assays on AcLAG-3Cv (for *A. californica* LAG-3C recombinant virus)-infected Sf9 cells (Fig. 1A) and their lack of binding to either noninfected Sf9 cells or wild-type AcNPv-infected Sf9 cells (data not shown). A polyclonal rabbit antiserum (anti-208B) raised against the 208B peptide was also reactive with AcLAG-3Cv-infected Sf9 cells, while the preimmune serum (PI) was not (Fig. 1A).

Both monoclonal (17B4 and 4F4) and polyclonal (anti-208B) anti-LAG-3 antibodies were tested for their ability to recognize a soluble recombinant LAG-3 protein (LAG-3S) corresponding to the three NH₂ terminus Ig-like LAG-3 domains. As shown by Western blot analysis of AcLAG-3Sv-infected Sf9 cell supernatant, they recognized a 43-kD protein band (Fig. 1B, lanes 2, 3, and 5). The translation of the 352-aa recombinant LAG-3S protein should result in a 38-kD molecule; this size difference may derive from the addition of N-glycans by the Sf9 insect cells (22). Together, these data demonstrated that immunization with the 208B peptide has allowed the generation of anti-LAG-3-specific reagents.

Initial experiments showed that both the 17B4 and 4F4 mAb reacted with PHA-activated PBMC (data not shown). The fine specificity of 17B4 was assessed in competition experiments using the 30-aa 208B peptide and a 28-aa LAG-3 unrelated peptide (termed NS). Preincubation of the 17B4 mAb with a 300-nM concentration of the 208B peptide abrogated 17B4 binding to PHA blasts (data not shown). Anti-LAG-3 mAb competitive binding to PHA blasts indicated that cell preincubation with saturating amounts of 17B4 abrogated 4F4 binding (data not shown). Together, these data confirmed that both antibodies recognize specifically the extra loop segment of the LAG-3 Ig-like first domain and showed that they are likely to react with the same or a closely related epitope, provisionally termed LAG-3.1.

Cellular Distribution of the LAG-3.1 Epitope. The kinetics

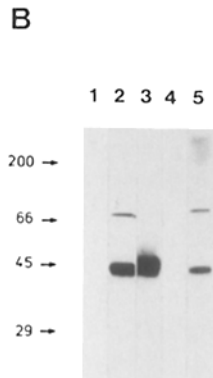
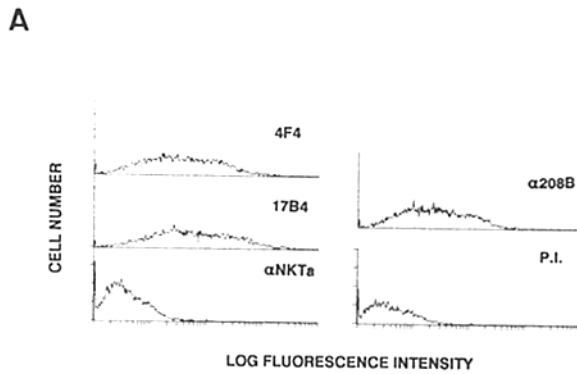


Figure 1. Reactivity of anti-LAG-3 antibodies. (A) Expression of LAG-3 on AcLAG-3Cv-infected Sf9 cells detected by indirect immunofluorescence. Cells were incubated with either anti-NKTα, 17B4, 4F4 plus goat anti-mouse serum or rabbit antiserum (anti-208B), preimmune rabbit serum (PI) plus goat anti-rabbit serum. Histograms represent \log_{10} red fluorescence intensity versus cell numbers. (B) Western blot analysis of AcLAG-3Sv-infected Sf9 cell supernatant collected at day 6 postinfection. The transfer membrane was incubated with anti-NKTα (lane 1), 4F4 (lane 2), 17B4 (lane 3), preimmune rabbit serum (lane 4), or anti-208B rabbit antiserum (lane 5).

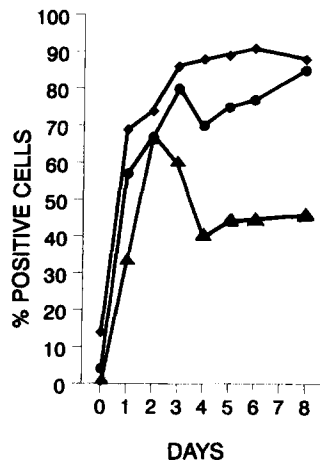


Figure 2. Kinetics of LAG-3.1 epitope expression. PBMC were stimulated by PHA and analyzed by indirect immunofluorescence with either 17B4 (▲), anti-CD25 (■), or anti-CD71 (●) mAbs.

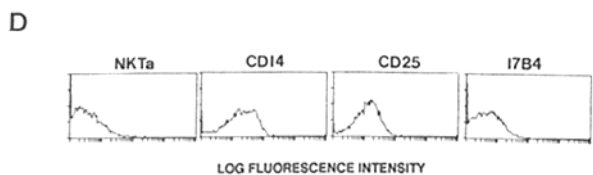
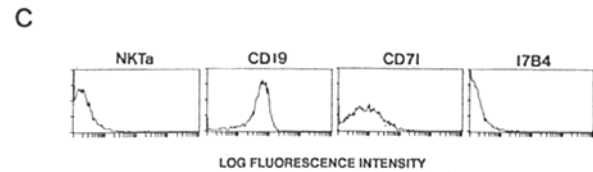
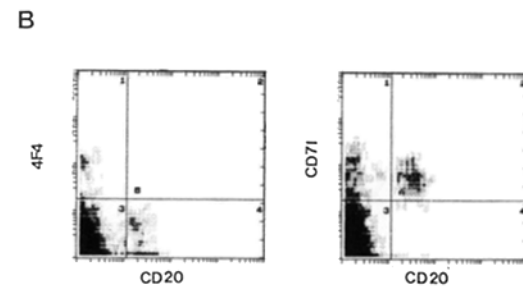
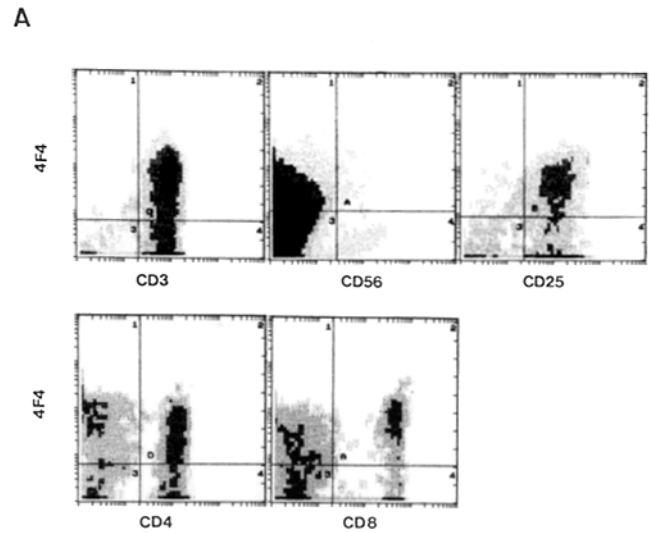


Figure 3. Phenotypic analysis of PBMC-activated cells by indirect immunofluorescence. (A and B) Double-color analysis of PHA-activated PBMC (A) or PWM-activated PBMC (B). x-axis shows \log_{10} green fluorescence intensity obtained with FITC goat anti-mouse serum specific for the corresponding antibody isotype (for each antigen). y-axis shows \log_{10} red fluorescence intensity obtained with phycoerythrin goat anti-mouse serum specific for the corresponding antibody isotype (for each antigen). (C and D) Tonsil cells (C) and LPS-activated monocytes (D) analyzed by indirect immunofluorescence. Histograms represent the number of cells versus the intensity of green fluorescence on a logarithmic scale. CD71 and CD25 are used as activation markers for tonsil cells and monocytes (47), respectively.

of LAG-3.1 epitope expression on PBMC was studied by indirect immunofluorescence from day 0 to 8 after PHA activation. Other activation antigens such as CD25 (IL-2R) and CD71 (transferrin receptor) were assessed in parallel (Fig. 2). At day 0, PBMC do not express the LAG-3.1 epitope. 17B4

binding was not detected at 6 h (data not shown), appeared at day 1, peaked at day 2 with >60% of the cells being positive, decreased progressively until day 8 (Fig. 2), and became virtually undetectable at day 11 (data not shown). Restimulation of PHA blasts at day 11 by addition of either IL-2 (25 and 250 U/ml), IL-4 (2.5 and 25 U/ml), or IFN- γ (10 and 1,000 U/ml) indicated that only IL-2 (at 250 U/ml) was able to reinduce the expression of the LAG-3.1 epitope (data not shown).

A series of long-term cultured IL-2-dependent clonal or polyclonal T cells, including MBA8, T2, R2, PH28, 1C1 (CD3⁺ TCR- α/β ⁺), TH6-4, BK (CD3⁺ TCR- γ/δ ⁺), as well as the NK cell lines F55IIIIE5 and SIB5 (CD3⁻ CD56⁺), were tested and all found to express (at varying density) the LAG-3.1 epitope (data not shown). Conversely, a series of transformed cell lines did not express the LAG-3.1 epitope: T cells (PEER, HSB2, REX, CEM, JURKAT, MOLT-4), B cells (LAZ388, LAZ461, RAJI, RAMOS, DAUDI, E418), and nonlymphoid cells (K562, HL-60, U-937, KG-1) (data not shown).

Taking advantage of the 4F4 mAb IgM isotype, coexpression by day 2 PHA blasts of the LAG-3.1 epitope and other molecules (CD4, CD8, CD3, CD56, or CD25) was studied by double-color immunofluorescence analysis. As shown in a representative experiment (Fig. 3 A), the LAG-3.1 epitope was expressed on a majority of CD3⁺ (63% of them being LAG-3⁺), CD25⁺ (53%), CD4⁺ (58%), or CD8⁺ (60%) lymphocytes. Approximately 3% of the PHA blasts expressing LAG-3.1 were CD3⁻. An equivalent fraction was CD56⁺. It is therefore likely that the corresponding cells were activated CD3⁻ CD56⁺ NK lymphocytes. Together, these results show that the LAG-3 molecule is present on a majority of activated T (CD4⁺ or CD8⁺) cells and on some NK cells at day 2 after PHA activation.

The LAG-3.1 epitope was not found on resting B cells (data not shown) nor on cultured (PWM-stimulated) peripheral blood B cells (Fig. 3 B) expressing the CD20 molecule; in these experiments the expression of CD71 (transferrin receptor) was used as a control for cell activation. The LAG-3.1 epitope was not found in tonsils (Fig. 3 C), where a large fraction of the B cells constitutively express CD71, in resting monocytes (data not shown), or LPS-activated monocytes (D). Together, these results confirm (1) that LAG-3 is selectively (i.e., T and NK cells as opposed to B cells and monocytes) expressed on a large fraction of activated PBMC.

Biochemical Characterization of the LAG-3 Protein. Immunoprecipitations were performed on ¹²⁵I-labeled day 2 PHA blasts, as well as on the IL-2-dependent NK clone, SIB5, which exhibited a higher LAG-3.1 epitope expression, as shown by immunofluorescence analysis (Fig. 4 A). SDS-PAGE analysis of the 17B4 immunoprecipitates from PHA blasts (Fig. 4 B, lane b) and SIB5 cells (C, lane b) resolved in nonreducing conditions a 42–45-kD doublet band in addition to the 70-kD LAG-3 molecule. Under reducing conditions, the doublet band appeared as a single 45-kD species (Fig. 4, B and C, lane e). Extensive preclearing of the lysates with antiactin mAb-coupled beads could rule out a possible contamination with

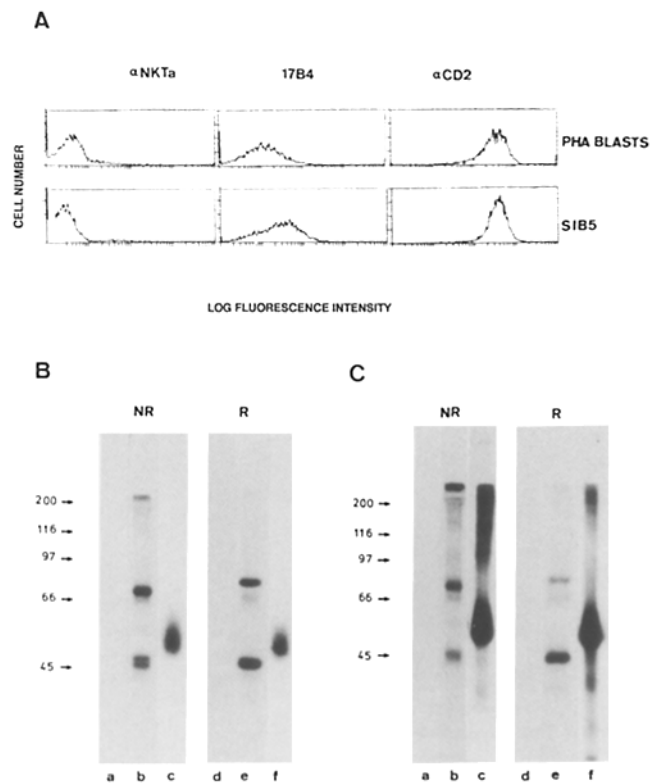


Figure 4. Immunoprecipitation of the LAG-3 protein from PHA blasts or SIB5 lysates. (A) LAG-3.1 epitope expression on PHA blasts or SIB5 cells as detected by indirect immunofluorescence. Histograms represent the number of cells versus intensity of fluorescence on a logarithmic scale. (B and C) SDS-PAGE analysis of the LAG-3 protein after immunoprecipitation with 17B4. PHA blasts (B) or SIB5 cells (C) were ¹²⁵I labeled and lysates were submitted to immunoprecipitation followed by analysis in SDS-PAGE under nonreducing (NR) and reducing (R) conditions. Immunoprecipitations were performed using anti-NKTa (lanes a and d), 17B4 (lanes b and e), or anti-CD2 (lanes c and f).

actin molecules. Immunoprecipitation positive and negative controls were performed by using anti-CD2 (lanes c and f) and anti-NKTa (lanes a and d) mAbs, respectively.

When PHA blast lysates were boiled in the presence of 0.5% SDS, 2 mM DTT (or with SDS alone; data not shown) before immunoprecipitation with 17B4, only the 70-kD protein was detected in SDS-PAGE (Fig. 5 A, lane 2), suggesting that the 70-kD protein but not the 45 kD proteins, is LAG-3 encoded. Western blot analysis (Fig. 5 B) of LAG-3⁺ PHA blasts (lane 2) and SIB5 (lane 3) lysates confirmed the presence of a similar 70-kD protein reactive with 17B4. This protein was not detected in LAG-3⁻ PBMCs (lane 1), E418 (an EBV-transformed B cell line; lane 5), and U937 (lane 6) lysates. Additional bands (68 and 60 kD; Fig. 5 B, lanes 2 and 3) reactive with 17B4 might correspond to immature, partially glycosylated forms of the LAG-3 protein since the expected molecular mass of the 470-aa LAG-3 protein backbone is 51 kD (1).

Together, the results of the immunoprecipitation experi-

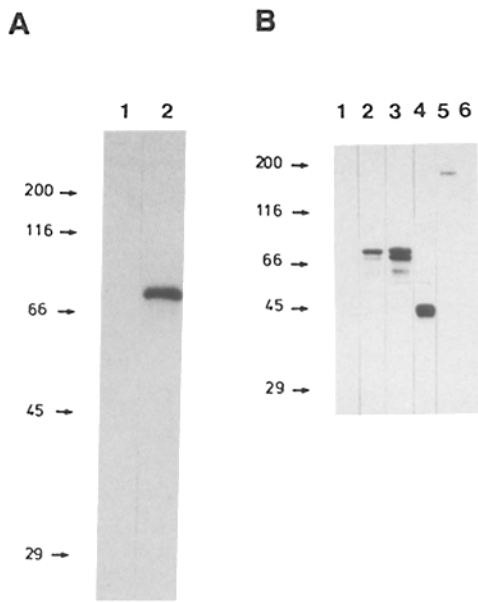


Figure 5. (A) Immunoprecipitation in denaturing conditions and SDS-PAGE analysis. ^{125}I -labeled PHA blast lysates were boiled in 1% SDS, 2 mM DTT, and subjected to immunoprecipitation with either anti-NKTa (lane 1) or 17B4 (lane 2). (B) Western blot analysis of T or NK cell lysates using 17B4. Samples included: PBMC (lane 1), PHA blasts (lane 2), SIB5 cells (lane 3), AcLAG-3S-infected Sf9 cells supernatant (lane 4; added as a positive control), E418 cell line (lane 5), U937 cell line (lane 6).

ments performed on activated T and NK cell lysates indicate that LAG-3 is likely to be noncovalently associated to one or several 45-kD molecule(s). One cannot exclude, however, that a LAG3.1 epitope-like structure may be present in a labile form (i.e., not recognized after denaturation) on distinct protein(s) migrating at 45 kD in SDS-PAGE analysis. Fur-

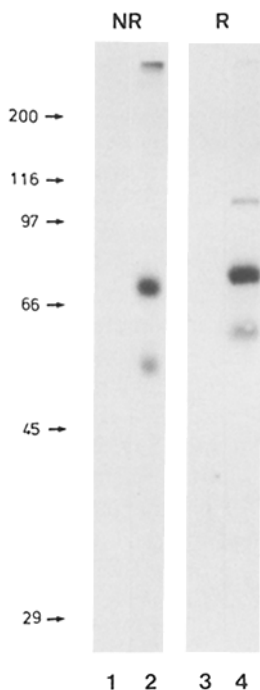


Figure 6. SDS-PAGE analysis of LAG-3 immunoprecipitated from transfected COS-7 cells. ^{125}I -labeled LAG-3-transfected COS-7 cell lysate was submitted to anti-NKTa (lanes 1 and 3) or 17B4 (lanes 2 and 4) immunoprecipitations. The immunocomplexes were analyzed under nonreducing (NR) and reducing conditions (R).

ther studies using reagents reacting with other segments of the LAG-3 protein will help to solve these questions.

Immunoprecipitation of LAG-3-transfected COS-7 cell lysates with 17B4 revealed a major band of 68–70 kD in both nonreducing and reducing conditions (Fig. 6, lanes 2 and 4). An additional 52–55-kD immunoprecipitated band was detected and may correspond to an immature form of the LAG-3 protein (in these experiments, a 30% COS-7 cell mortality was observed before ^{125}I labeling) or to the association with a molecule distinct from that observed in activated T and NK lymphocyte lysates.

The two proteins (70 and 45 kD) immunoprecipitated from PHA blast lysates were extracted from the gel and independently submitted to N-glycosidase-F treatment. Reduction in size was observed for both species from 70 to 60 kD (Fig. 7, lanes 1 and 2) and from 45 to 40 kD (lanes 3 and 4), indicating that molecules are glycosylated. The difference seen between the apparent mass of LAG-3 (60 kD) after N-glycosidase-F treatment and the predicted molecular mass of the mature protein backbone (51 kD) may be related to incomplete N-deglycosylation (four potential N-linked glycosylation sites in the LAG-3 sequence) or to other posttranslational modifications.

B Lymphocyte Adhesion to LAG-3-transfected COS Cells. We used the high-level expression cellular system based on COS-7 cell transfection with recombinant CDM8 vectors (23). This system has proved to be the most convenient to study binding of transiently expressed wild-type and mutant CD4 with low affinity to HLA class II and with high affinity to gp120 (8, 24, 25). Transfections of COS-7 cells were performed in parallel with the CDM8-LAG-3 and CDM8-CD4 recombinant vectors. Cell surface expression, rosette formation with class II-bearing B lymphocytes, and gp120 binding were assayed. A LAG-3 insert cloned in the reverse orientation, termed CDM8-GAL, was used as negative control.

As shown by flow cytometry analysis with 17B4 (Fig. 8 A), 30–40% COS-7 cells assessed on day 3 were found to express LAG-3 specifically. COS cells transfected with the reverse construction GAL were not reactive with 17B4. Adhesion of the HLA class II-expressing Burkitt lymphoma line,

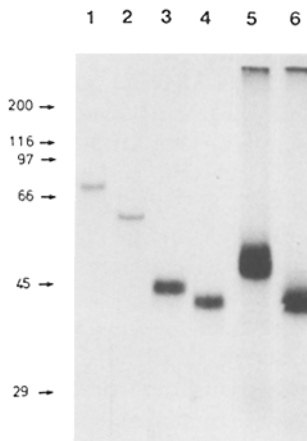


Figure 7. N-Glycosidase-F treatment of the proteins immunoprecipitated with 17B4. Both 17B4-reactive proteins (70 and 45 kD) were isolated independently and subjected to deglycosylation. Samples were analyzed by SDS-PAGE under reducing conditions: 70-kD LAG-3 protein sham cleaved (lane 1) or N-Glycosidase-F cleaved (lane 2); 45-kD LAG-3-associated protein sham (lane 3) or N-Glycosidase-F cleaved (lane 4); CD2 protein sham- (lane 5) or N-glycosidase-F cleaved (lane 6).

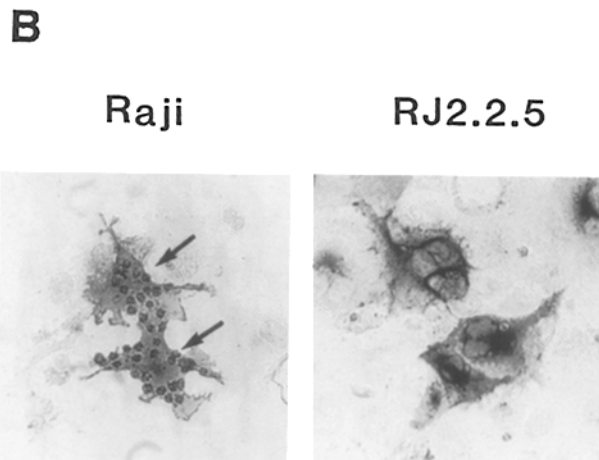
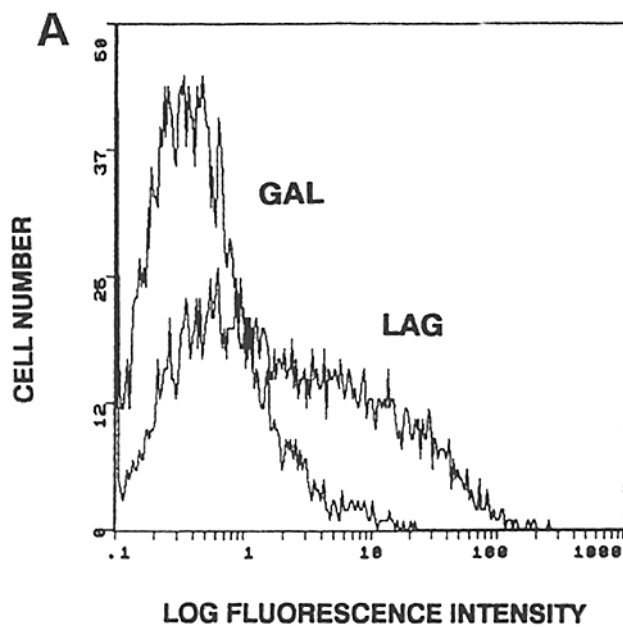


Figure 8. Transient membrane expression of LAG-3 in COS-7 cells and rosette formation between LAG-3-expression COS cells and B lymphocytes expressing HLA class II molecules. (A) Flow cytometry analysis of anti-LAG-3 17B4 mAb binding to COS cells transfected with the recombinant CDM8-LAG-3 vector (LAG). The CDM8-GAL construct, as described in Materials and Methods, was used as a negative control (GAL). (B) Specific adhesion of the HLA class II-positive, Raji (arrows), but not of the HLA class II-negative, RJ2.2.5, B lymphocytes to COS cells that express LAG-3, as detected by immunoperoxidase staining with the 17B4 mAb.

Raji, occurred only with LAG-3-expressing COS cell ($23 \pm 3\%$, $n = 26$), as detected by immunoperoxidase staining with the 17B4 antibody (Fig. 8 B, arrows). No rosettes were observed with the HLA class II-negative mutant of Raji, RJ2.2.5

(26) (Fig. 8 B). COS cells transfected with the GAL construct did not form rosettes with Raji (data not shown). Rosettes of Raji cells formed with LAG-3- and CD4-positive (not shown) COS cells were morphologically indistinguishable. A series of cells expressing high levels of HLA class II antigens (Daudi, the EBV-transformed cell lines E418, and ALF, the latter deriving from an LFA-1-negative immunodeficient patient; reference 27) were found to form rosettes, while a series of class II-negative cells, K562, HSB2, BCH (28), and the TC line derived from a HLA class II-immunodeficient patient (29), did not (data not shown). Together these results suggested that rosette formation was induced by the interaction of HLA class II and LAG-3 molecules.

Rosettes between LAG-3-positive COS cells and Raji were specifically inhibited in a dose-dependent manner by the LAG-3-specific 17B4 mAb (Fig. 9 A) and by two antibodies specific for HLA class II molecules: the monomorphic anti-HLA-DR mAb D1.12 (14) and the 9.49 mAb specific for the three HLA class II isotypes (15) (Fig. 9 B). Since anti-class II antibodies are known to induce aggregation of Raji B lymphocytes, as the result of activation of LFA-1-dependent (30) or -independent (31) cell adhesion pathways, it raised the possibility that aggregation might interfere with the inhibitory properties of these antibodies. Raji cells were fixed with 0.5% paraformaldehyde before incubation with anti-class II antibodies. This treatment did not modify antibody binding to Raji (data not shown) and suppressed the aggregation process. In these conditions both anti-class II mAbs specifically inhibited the rosette formation (Fig. 9 B). The 9.49 mAb induced stronger rosette inhibition than D1.12, indicating that HLA class II isotypes other than DR (i.e., DQ and DP) may interact with LAG-3, as described for CD4 (5). In contrast, no inhibition was observed with the isotype-matched (IgG1) anti-CD4 mAb, OKT4E (Fig. 9 A), which is a potent inhibitor of rosettes between CD4-positive COS cells and Raji, as previously described (8), nor with the anti-ICAM-1 mAb, 8F5 (16), and the anti-LFA-1 CD11a-specific mAb, 25.3.1 (Fig. 9 B), which are known to inhibit strongly cell adhesion mediated by LFA-1/ICAM-1 interactions. Similarly, the HLA class I-specific mAb, W6.32 (32), did not induce rosette inhibition (data not shown). These results demonstrate that cellular adhesion is directly dependent upon specific LAG-3/HLA class II interaction.

Since LAG-3 and CD4 are closely related, we have also examined the ability of the LAG-3 protein to bind the HIV gp120 protein. In contrast to CD4, binding of iodinated recombinant HIV gp120 to LAG-3-transfected COS cells, as measured by a sensitive radioimmunoassay (24), was undetectable (data not shown).

Discussion

We have generated two anti-LAG-3 mAbs after mice immunization with a 30-aa peptide coupled at its NH₂ terminus to tetanus toxoid. These reagents recognize specifically both membrane expressed and soluble recombinant LAG-3 molecules produced in a baculovirus expression system. The two antibodies are likely to react with the same or a closely

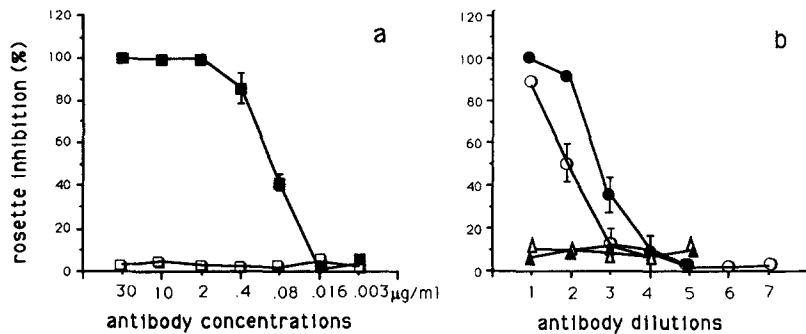


Figure 9. Specific binding inhibition of class II-bearing B lymphocytes to LAG-3-positive COS cells. Different concentrations of antibodies were incubated 30 min before rosette formation with LAG-3-transfected COS cells (a), or with Raji B lymphocytes (b). Antibody to LAG-3 was 17B4 (■); to CD4, OKT4E (□); to HLA-DR, D1.12 (○); to HLA-DR, -DQ, and -DP, 9.49 (●); to ICAM-1, 8F5 (▲); and to LFA-1 CD11a, 25.3.1 (△). Rosettes were allowed to form in the presence of the inhibitors and quantitated, as described (8). Results are expressed as the rosette inhibition percentage of two or more experiments ± SD, as described in Materials and Methods. Antibody amount is expressed as -log₅ (dilution ratio) indicated as the number of 1/log₅ increasing dilutions, when used as ascitic fluids (b).

related epitope, provisionally termed LAG-3.1, exposed on the extra loop segment of the LAG-3 Ig-like first domain. Using these antibodies, we demonstrate here that a 70-kD molecule carrying the LAG-3.1 epitope is selectively expressed on the surface of activated T and NK cells as an "intermediate" activation antigen (33). Note that in Northern blot analysis, LAG-3 mRNA expression could not be clearly detected before day 3 in PHA-stimulated lymphocytes (1).

Peptide immunization may lead in some instances to the generation of antibodies reacting with the native form of the corresponding protein (34). The present series of experiments show that two murine mAbs (17B4 and 4F4) as well as a rabbit antiserum generated by peptide immunization are able to recognize the native LAG-3-encoded protein in immunofluorescence assays. The generation of such antibodies may have been facilitated by the structure of the immunizing peptide, given its length (30 aa) and the fact that it includes 10 prolines that are known to favor the induction of protein-reactive antibodies (35). In any case, the present results confirm that at least an epitope (LAG-3.1) on the extra loop is exposed to the solvent on the surface of the LAG-3 Ig-like first domain (1).

Since LAG-3 and CD4 genes have been considered to derive from a common evolutionary ancestor (1), initial studies

on the LAG-3 biological function(s) have been designed to investigate whether CD4 and LAG-3 share common ligands: HLA class II antigens and HIV gp120. The inability of LAG-3 to bind HIV gp120 could be expected because of the limited amino acid conservation (4/18 residues) with the CD4 gp120 binding site and because it seems purely fortuitous that HIV has subverted the human CD4 molecule (and not the murine or the rat CD4) for its use (36). To analyze potential LAG-3/HLA class II interactions, we used a quantitative cellular adhesion assay (8), based on the transient expression upon transfection of high levels of the LAG-3 protein by COS-7 cells. We were able to demonstrate the formation of rosettes between LAG-3-transfected COS-7 cells and HLA class II-bearing B lymphocytes, which is specifically dependent on LAG-3/class II interaction as shown in blocking experiments using 17B4 and anti-class II mAbs.

These observations raise the question of the respective localization of the class II binding sites for LAG-3 and CD4. We (8, 24, and unpublished data) and others (25) have proposed that the HLA class II and gp120 binding sites (Fig. 10, arrows) were largely overlapping on CD4. On the other hand, using a different functional assay, others have proposed that both sites were separable (37-39), defining three key residues (Fig. 10, arrowheads) within exposed loops from CD4

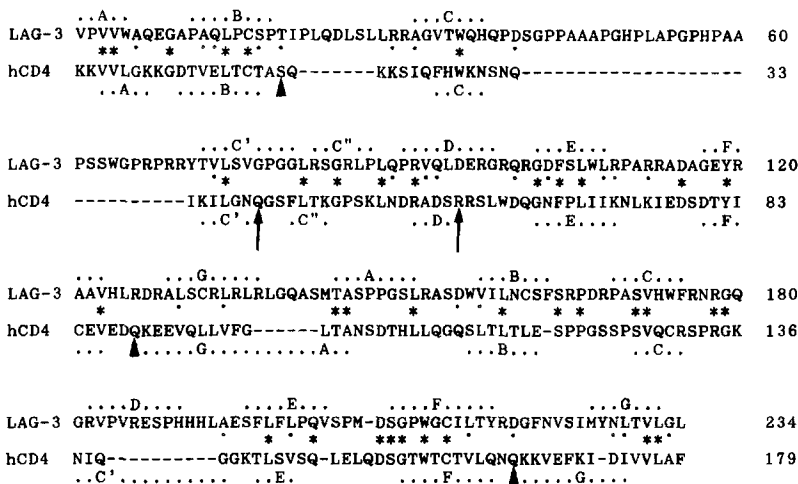


Figure 10. Alignments of LAG-3 and human CD4 amino acid sequences in domains 1 and 2. Identities (*), similarities (°), and gaps (-). The dotted lines above and under the sequences indicate the positions of putative or determined β strands of LAG-3 and CD4 (48, 49), respectively. The putative HLA class II binding sites on CD4 are marked by arrows (24, 25) and closed arrowheads (37-39).

domains 1 and 2, and located on the sheet opposite the gp120 binding site. None of these CD4 putative class II binding sites are conserved in LAG-3 (Fig. 10), suggesting that different LAG-3 regions might be involved in this function. This could be expected since the sequence similarity in human CD4 and LAG-3 domains 1 and 2 is only 17%, excluding the LAG-3 30-aa extra loop, which has no equivalent in CD4. Template modeling together with site-directed mutagenesis will help towards a better definition of the class II binding sites.

The demonstration that LAG-3 and CD4 proteins share a common ligand (i.e., HLA class II antigens) is of great importance to design further investigations for determining the function of this molecule. In vitro HLA class II-dependent cellular responses, such as mixed lymphocyte cultures, varidase (a mixture of streptokinase/streptodornase antigens; reference 40), or OKT3 (41)-induced proliferations were studied in the presence of purified anti-LAG-3 mAb (17B4) or control antibodies. The addition of 17B4 had no effect on [³H]TdR incorporation by proliferating lymphocytes in these assay systems when compared with control antibodies (data not shown). The absence of inhibition of HLA class II-dependent cellular responses by 17B4 may relate to the complexity of cellular interactions known to involve other molecules expressed on activated lymphocytes (e.g., CD4/HLA class II, LFA-1/ICAM-1, or CD2/LFA-3). In addition, these initial experiments designed to assess primary in vitro stimulation might be inappropriate to detect inhibitory effects of anti-LAG-3 mAb because cells are already committed to proliferate when the LAG-3 protein is expressed at the cell

surface. In this respect, studies will have to test whether LAG-3⁺-activated T cells may be inhibited in secondary or subsequent class II-dependent responses. Similarly, CD4⁻ T lymphocytes mediating class II-restricted regulatory or effector functions have been occasionally characterized (42-46), and studying such infrequent cell types would represent a favorable experimental situation to assess LAG-3/class II interaction functions.

LAG-3 may be effective in contributing to the stabilization of activated T cell interactions with macrophages, dendritic cells, or activated B cells, which are known to express high numbers of class II molecules on their surface. The present results, which show that LAG-3 is expressed on activated CD4⁻ T cells, suggest that this molecule may also stabilize cell-cell contacts in T cell responses that are not restricted by a TCR/peptide/class II interaction (e.g., class I-restricted CD8⁺ TCR- α/β ⁺ and most TCR- γ/δ ⁺ cells). In addition, the demonstration of the expression of LAG-3 on activated NK lymphocytes, which are generally known as cells expressing a "non-MHC-requiring cytotoxicity," suggests that it may also be of interest to consider a potential contribution of MHC class II molecules in these TCR-independent cytotoxic reactions.

In conclusion, the potential role of LAG-3/HLA class II interactions in the development of lymphocyte effector functions, in the regulation of immune responses, and during thymic ontogeny will have to be assessed in light of the present findings.

We thank Frédéric Troalen for the synthesis of the 208B peptide and Alain David for the purification of the 17B4 mAb. We also thank Barbara Grosppierre (INSERM U132, Paris) for providing the ALF and TC cell lines, Patricia Rao (Johnson Pharmaceutical Research Institute, Raritan, NJ) for providing antibodies, and Marie-Paule Kiény and Jean-Pierre Lecocq (Société Transgène, France) for recombinant gp120.

This work was supported by INSERM, CNRS, and in part by grants from Association pour la Recherche sur le Cancer to C. Auffray and F. Triebel, from Agence Nationale de Recherche sur le SIDA to C. Auffray, and from ARES-SERONO to F. Triebel.

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Received for publication 3 February 1992 and in revised form 8 April 1992.

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